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Expression of endothelin-3 mRNA along rat nephron segments using polymerase chain reaction

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Expression of endothelin-3 mRNA along rat nephron segments using polymerase chain reaction. Endothelin (ET) is now known to be a family of three distinct peptides. Although many reports have studied the renal action of ET-1, comparatively little is known concerning ET-3. We previously reported that ET-1 mRNA is expressed in glomerulus (Glm) and inner medullary collecting duct (IMCD). In this study, microlocalization of mRNA coding ET-3 was carried out in the rat kidney using a reverse transcription and polymerase chain reaction (RT-PCR) assay of individual microdissected renal tubule segments along the nephron, Glm, vasa recta bundle, and arcuate arteries. Large signals for ET-3 PCR product were detected in proximal convoluted and straight tubules, cortical collecting duct, and outer medullary collecting duct. Glm, IMCD, and vasa recta bundle also expressed relatively large amounts of ET-3 mRNA. Small signals were found in medullary thick ascending limb, inner medullary thin limb, and arcuate artery. We detected ET-3 protein in tubule suspensions from cortex, outer medulla, and inner medulla of rat kidney. Furthermore, incubation with TGF-β did not change ET-3 PCR signal, whereas ET-1 PCR signal was increased significantly by exposure to TGF-β in Glm and IMCD. Thus, ET-3 and ET-1 are distributed differently along the nephron and are regulated in different manners. This suggests that ET-3 and ET-1 may affect kidney functions in different ways.

Endothelin (ET), initially described as a 21-amino-acid vasoactive peptide secreted by cultured vascular endothelial cells [1], is now known to be a family of three distinct peptides [2]. ET-1 differs in structure from endothelin-2 (ET-2) and endothelin-3 (ET-3) by two and six amino acid residues, respectively [2]. Recently, two distinct ET receptors were described. A-type ET receptor binds ET-1 with two- to 10-fold higher affinity than ET-2 and with more than 100-fold higher affinity than ET-3 [3]. B-type ET receptor (ET-BR) has similar affinities for all three peptides [4].

The kidney has been reported to be the production and action site of ETs. We reported that ET-1 mRNA and protein are present in glomerulus and inner medullary collecting duct (IMCD) [5]. Although many reports have studied the renal action of ET-1, comparatively little is known concerning ET-3. Recently, several reports demonstrated that ET-3 plays some role in maintaining glomerular function and renal blood flow (RBF) [6, 7]. We previously reported that ET-BR is widely expressed along the nephron segments, and found especially high levels in glomerulus and collecting ducts [8]; ET-3 could modulate renal functions via ET-BR.

Recently, Firth and Ratcliffe reported that relatively large amounts of ET-3 mRNA were expressed in the kidney [9]. ET-3 peptide was also detected in the kidney by Hemsen and Lundberg using HPLC [10]. However, these studies did not determine which nephron segments produce ET-3. The kidney is a heterogenous organ, and thus information on the precise localization and regulation of ET-3 mRNA expression seems necessary to understand the renal actions of ET-3. ET-3 production was reported in primary cultures of renal tubule cells obtained from proximal tubule, medullary thick ascending limb, cortical collecting duct, and IMCD [11]. It is not known whether native renal tubules express ET-3 mRNA. It is also not known whether ET-3 and ET-1 are regulated by similar mechanisms. Therefore, we investigated the effects of transforming growth factor (TGF)-β, which is known to induce ET-1 mRNA, on the expression of ET-3 mRNA.

Recently, Moriyama et al [12] introduced a new method for measuring relative levels of specific mRNA in microdissected glomeruli or renal tubules using the polymerase chain reaction (PCR) coupled to reverse transcription (RT-PCR). In the present study, we employed this RT-PCR technique to investigate the precise localization and regulation of ET-3 in microdissected renal tubules, glomeruli, arcuate artery, and vasa recta bundle.

Methods

Microdissection of nephron segments

Nephron segments were microdissected from male Sprague-Dawley rats weighing 75 to 100 g as previously described [12, 13]. The kidney was perfused initially with 10 ml of ice-cold dissection solution (solution 1), and then with 10 ml of the same solution containing 1 mg/ml collagenase (collagenase solution) (type I, 300 U/mg, Sigma Chemical Co., St. Louis, Missouri, USA) and 1 mg/ml bovine serum albumin (Sigma). The dissection solution (solution 1) contained the following (in mM): 135 NaCl, 1 Na_2SO_4, 1.2 $MgSO_4$, 5 KCl, 2 $CaCl_2$, 5.5 glucose, and 5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4. The left kidney was removed and a coronal section was made that contained the entire corticopapillary

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axis. This section was cut into four pieces: cortex, outer medulla, outer 25% of inner medulla, and inner 75% of inner medulla. These pieces were incubated in collagenase solution for 30 minutes (cortex and outer medulla) or 40 minutes (inner medulla) at 37°C in a shaking water bath. Next, tissues were transferred to the dissection solution containing 10 mM vanadyl ribonucleotide complex (Life Technologies, Inc., Gaithersburg, Maryland, USA), a potent RNase inhibitor.

We microdissected the following structures: glomerulus (Glm), proximal convoluted tubule (PCT), proximal straight tubule (PST), inner medullary thin limb (IMTL), medullary thick ascending limb (MTAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), the initial part of inner medullary collecting duct (iIMCD), the terminal part of inner medullary collecting duct (tIMCD), arcuate artery, and vasa recta bundle (VR bundle) [12, 13].

Microdissected renal structures were washed free of contaminating debris and vanadyl ribonucleotide complex in a separate wash dish containing 10 ml of the dissection solution. Generally, a 2 mm length of tubule or arcuate artery was transferred into the appropriate RT-PCR reaction tube, containing 10 μl of ice-cold dissection solution containing > 1 U/μl of human placental RNase inhibitor (Boehringer-Mannheim, GmbH, Mannheim, Germany) and 5 mM dithiothreitol (DTT, Sigma).

Reverse transcription

Reverse transcription (RT) was performed using a cDNA synthesis kit (Boehringer-Mannheim). The RNase-inhibitor solution was removed, and 9 μl of 2% Triton X-100, containing >1 U/μl of RNase inhibitor and 5 mM DTT, was added to permeabilize the cells. RT components were added to the reaction tubes as described previously [12, 13]: 4 μl of buffer I, 1 μl of RNase inhibitor, 2 μl of deoxynucleotide mixture (final concentration was 20 nmol of dATP, dCTP, dGTP, and dTTP), 2 μl of random primer (0.04 A_{260} unit of $p(dN)_6$ per reaction), and 2 μl of avian myeloblastoma virus reverse transcriptase. Reaction tubes were incubated at 42°C for 60 minutes in the Programmed Tempcontrol System (Astec, Tokyo, Japan). At the end of the incubation period, the reaction was stopped by heating at 90°C for five minutes.

Polymerase chain reaction

PCR was performed using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, Connecticut, USA), with rat ET-3-specific primers prepared on a DNA synthesizer (Applied Biosystems, Tokyo, Japan). We designed specific primers 20 to 25 nucleotides in length with 50 to 60% GC composition. ET-3 primer 1 (antisense) was defined by bases 479-499, and primer 2 (sense) encompassed bases 23-42 [14]. The sequence of ET-3 primer 1 was 5′-GCTGGTGGACTTTATCTGTCC-3′; primer 2 was 5′-TTCTCGGGCTCACAGTGACC-3′. The predominant cDNA amplification product was predicted to be 477 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 61-80 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5′-CCTGCACAGCCTGGAAATGC-3′ [14].

The primers for ET-1 were defined by the following cDNA base sequences [15]: primer 1 (antisense), bases 675-699, sequence, 5′-AAGATCCCAGCCAGCATGGAGAGCG-3′; primer 2 (sense), bases 157-181, sequence, 5′-CGTTGCTCCTGCTCCTCCTTGATGG-3′. The cDNA amplification product was predicted to be 543 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 302-321 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5′-CAAAGACCACAGACCAAGGG-3′.

RT and PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as a positive control. The primers were defined by the following cDNA base sequences [16]: primer 1 (antisense), bases 794-813, sequence, 5′-AGATCCACAACGGATACATT-3′; primer 2 (sense), bases 506-525, sequence, 5′-TCCCTCAAGATTGTCAGCAA-3′. The cDNA amplification product was predicted to be 309 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 307-326 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5′-ACCGCTTCCATGGCCCTGAT-3′. When GAPDH was used as a positive control, reverse transcription was performed, 20 μl samples were then divided into 15 μl for ET-1 or ET-3 and 5 μl for GAPDH. The volume was adjusted to 20 μl with sterile water. Next, parallel PCR reactions were run with each set of primers.

To carry out the PCR, 80 μl of a PCR master mix were added to each tube directly. Fifty picomoles each of primers 1 and 2 were used per reaction. Deoxynucleotides were added to a final concentration of 0.20 mM each. Reaction buffer (10×) was diluted (1/10) to have a final composition of: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin, and 2.5 units of Taq DNA polymerase. The tubes were placed in the Programmed Tempcontrol System which was programmed as follows: (a) incubation at 94°C for three minutes (initial melt); (b) 30 cycles of the following sequential steps: 94°C for one minute (melt); 60°C for one minute (anneal); 72°C for three minutes (extend). (c) Finally, incubation was done at 72°C for seven minutes (final extension). We also tested specifically for the possibility of a PCR "amplification plateau." We examined the nature of the amplification for ET-3 and ET-1 by performing RT-PCR for 0, 10, 20, 30, 40, and 45 cycles on samples of 2 mm lengths of OMCD, 10 mm lengths of IMCD, or 10 Glm. The reaction was as described above, but the PCR mixture was "spiked" with [^{32}P-α]dATP and [^{32}P-α]dCTP. After electrophoresis, the ET-3 and ET-1 bands were excised from each lane and ^{32}P incorporation into the amplification product was quantitated by scintillation counting of the agarose gel piece. Plotting the number of cycles versus radioactive counts confirmed that PCR amplification was log-linear until 40 cycles for 2 mm lengths of OMCD, 10 mm lengths of IMCD, and 10 Glm (data not shown).

PCR product analysis

Ninety microliters of the total reaction volume was ethanol-precipitated [17]. The PCR products were size-fractionated by agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator (Funakoshi, Tokyo, Japan).

For Southern blot analysis, gels were denatured, neutralized,

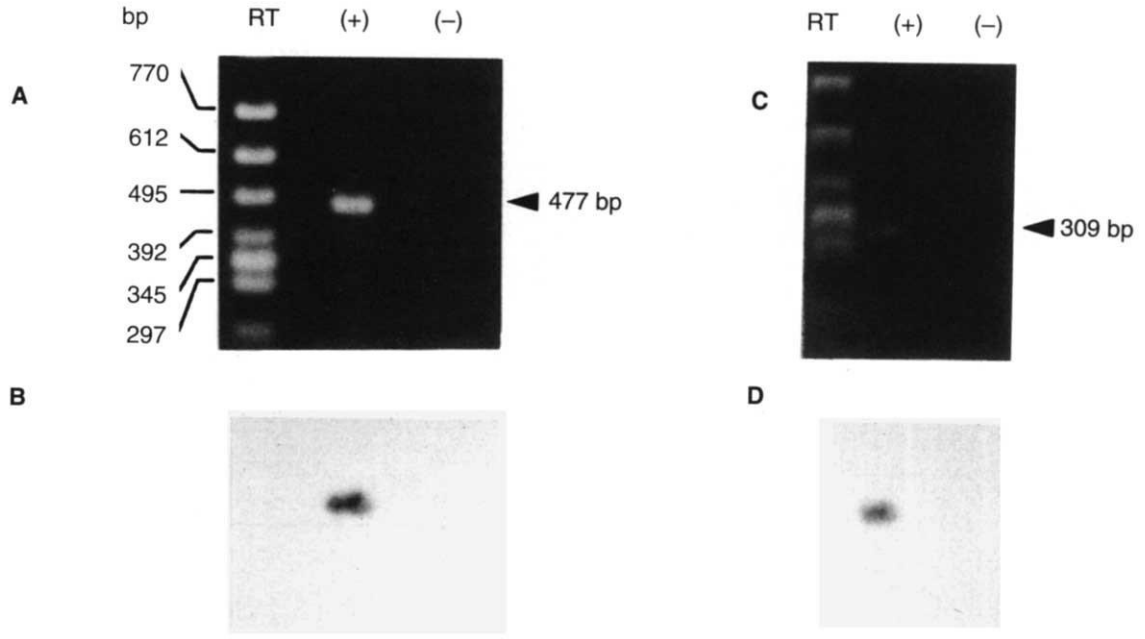

Fig. 1. *Effect of reverse transcription (RT) on ET-3 and GAPDH mRNA amplification.* **(A)** Ethidium bromide stained agarose gel for ET-3. The left lane is DNA size marker. **(B)** Autoradiogram of corresponding Southern blot. The blots were probed with a ^{32}P-labeled oligonucleotide that localized between ET-3 primers. **(C)** Ethidium bromide stained agarose gel for GAPDH. **(D)** Autoradiogram of corresponding Southern blot. The blots were probed with a ^{32}P-labeled oligonucleotide that localized between GAPDH primers. We obtained the same results from 3 experiments from 3 rats.

and blotted onto a nitrocellulose filter (Funakoshi). The synthetic oligonucleotide probes were end-labeled with ^{32}P as described previously [12, 13]. Prehybridization/hybridization washes were as previously described [12, 13].

To confirm that the PCR products were really ET-1 and ET-3 cDNAs, the PCR products were sequenced. PCR products from glomeruli were separated by gel electrophoresis. PCR products were subcloned into pCR™ vector (Invitrogen, San Diego, California, USA), then sequenced using the dideoxynucleotide chain-termination reaction. To monitor the presence of cross-contamination between other segments or debris, we have previously conducted a study which used aldose reductase mRNA from nephron segments as a marker [8]. Distribution of aldose reductase mRNA, which was reported present in Glm, IMCD, and IMTL [12], was studied to rule out the possibility that other segments were contaminated by IMCD or Glm. We did not detect any aldose reductase band in other segments [8]. This suggested that there was no cross-contamination between these segments. We also performed RT-PCR for ET-3 and ET-1 in the absence of template, to monitor the presence of contamination. We did not detect any band in the absence of template (data not shown).

ET-3 and ET-1 mRNA expressions in Glm and IMCD incubated with TGF-β

To investigate the effect of TGF-β on ET-1 and ET-3 mRNA expressions, 10 Glm or 10 mm lengths of microdissected IMCD were incubated in dissection solution described above, with or without TGF-β (16 ng/ml) (Collaborative Research, Inc., Bedford, Massachusetts, USA) in the presence of 0.5% fetal calf serum (FCS) (Life Technologies, Inc., Gaithersburg, Maryland, USA) for six hours at 37°C with oxygen supply. In our previous study [5], we have observed that ET-1 production was increased during 16 hours in this incubation condition; thus, we felt certain that Glm and IMCD are viable for six hours in this condition.

Relative quantitation of mRNA levels from autoradiographs

The PCR product for ET-3 was detected from several nephron segments. The relative amount of PCR product was determined by densitometric scanning of autoradiographs using a laser densitometer (Hoefer Scientific Instruments, San Francisco, California, USA). Each RT-PCR experiment included one or more OMCD samples. For relative quantitation, we used the densitometry value from the OMCD samples as an internal standard (100%) and calculated the percentage of the OMCD value for each segment.

To test the relationship between the quantity of starting material and that of amplification product of 30 cycles PCR as reflected by densitometric analysis, we compared the densitometry value obtained from the amplification product from several lengths of OMCD (0.2 to 2.0 mm, $N = 12$). We also compared the densitometry value obtained from the amplification product of 30 cycles PCR from several lengths of IMCD (1.0 to 20.0 mm, $N = 12$), and several numbers of Glm (1 to 20 Glm, $N = 12$) to test the relationship between the quantity of starting material and that of amplification product.

Measurement of ET-3 production in tubule suspensions and microdissected nephron segments

Kidneys from male Sprague-Dawley rats weighing 150 g to 250 g were used to make tubule suspensions as previously described [5]. In brief, after perfusion with solution 1, the left kidney was divided into three parts: cortex, outer medulla, and

inner medulla. After mincing, each part was incubated in solution 1 with 0.1% BSA and 0.1% collagenase (Sigma Chemical) in the presence of 100% O_2 for 30 minutes (for cortical tubule suspensions) or 45 minutes (for outer and inner medullary tubule suspensions). Microdissected nephron segments were Glm, PCT, MTAL, OMCD, and IMCD. Two hundred Glm or 100 mm lengths of tubules were used as one sample to determine ET-3 production. The final volume of each sample was 300 μl; the tubule suspension or nephron segments were homogenized using an ultrasonicator (Central, Tokyo, Japan), and centrifuged for 10 minutes at 3,000 rpm at 4°C. The supernatant was frozen until ET-3 was assayed. ET-3 was extracted using Sep-Pak C_{18} cartridges (Water Assoc., Milford, Massachusetts, USA). The RIA of ET-3 was performed using ET-3-specific antibody (Peninsula Laboratories, Belmont, California) after extraction of samples [18].

Statistics

The results were expressed as means ± SEM. The differences were tested using analysis of variance. $P < 0.05$ was considered significant.

Results

Effect of reverse transcription on ET-3 and GAPDH mRNA amplification

With reverse transcription, we detected a clear single band, which was the predicted size of 477 bp for ET-3, from OMCD (Fig. 1, A and B). When the PCR procedure was carried out in the absence of reverse transcriptase, the 477 bp band was not seen and there was no other recognizable band. With reverse transcription, we detected a clear single band, which was the predicted size of 309 bp for GAPDH, from OMCD (Fig. 1, C and D). When the PCR procedure was carried out in the absence of reverse transcriptase, the 309 bp band was not seen and there was no other recognizable band. This indicated that the 477 bp band and the 309 bp band originated from mRNA, not from genomic DNA.

Relationship between the quantity of starting material and that of amplification product

We compared amplification products of 30 cycles PCR from several lengths of OMCD for ET-3, assigning the 2 mm OMCD an arbitrary densitometric unit of 100 (Fig. 2). Linear regression analysis showed high correlation between densitometry values and the length of each tubule for ET-3 ($r = 0.94$; Fig. 2A). We also compared the densitometry value obtained from the amplification product of 30 cycles PCR from several lengths of IMCD (1.0 to 20.0 mm, $N = 12$), and several numbers of Glm (1 to 20 Glm, $N = 12$) to test the relationship between the quantity of starting material and that of amplification product, assigning the 20 mm lengths of IMCD or 20 Glm an arbitrary densitometric unit of 100. Linear regression analysis showed high correlation between densitometry values and the length of each tubule ($r = 0.93$; Fig. 2B) or the number of Glm ($r = 0.94$; Fig. 2C).

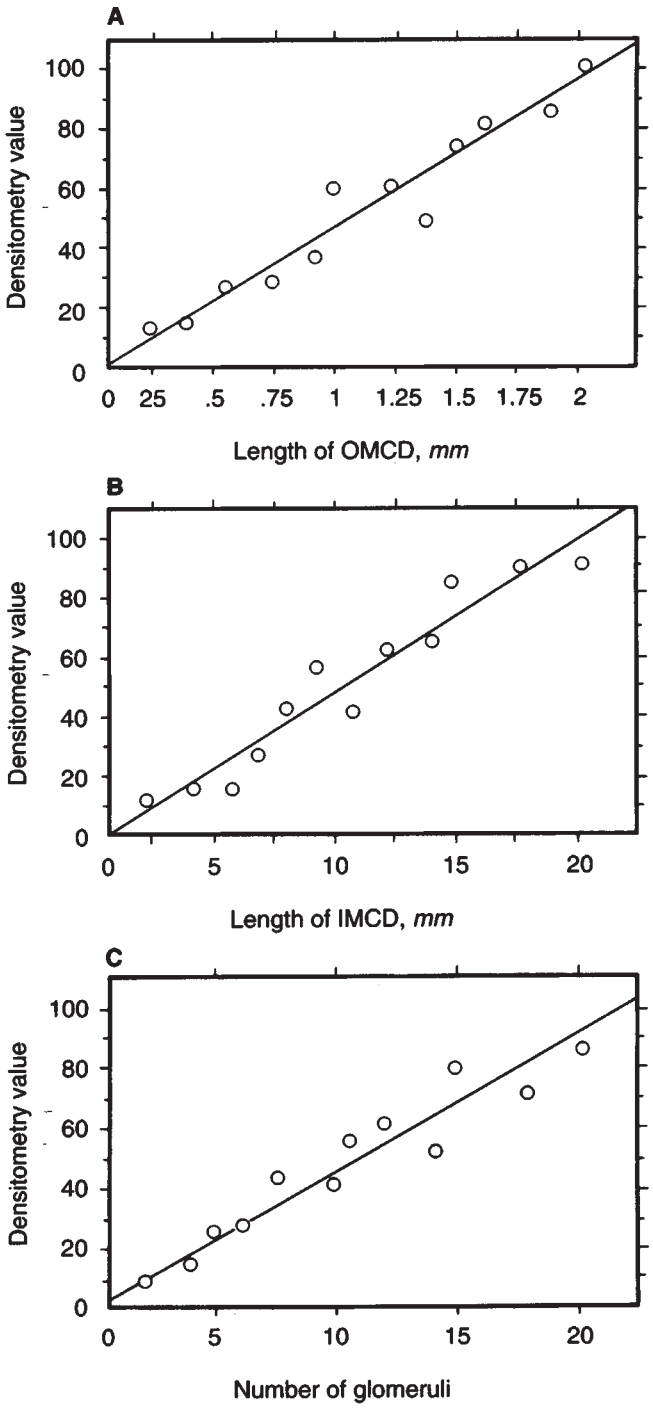

Fig. 2. *Relationship between the quantity of starting material (tubular length or glomerular number) and the resulting amplification product of 30 cycles PCR for ET-3 mRNA.* An arbitrary value of 100 was assigned to the sample representing 2 mm OMCD (tubule number = 12, from one rat, $r = 0.94$) (A). An arbitrary value of 100 was assigned to the sample representing 20 mm IMCD (sample number = 12, from one rat, $r = 0.93$). (B). An arbitrary value of 100 was assigned to the sample representing 20 Glm (sample number = 12, from one rat, $r = 0.94$). (C).

Distribution of ET-3 mRNA in microdissected structures

Each reaction was performed using either the 2 mm of tubule length, 5 Glm, a single VR bundle, or a 2 mm length of arcuate artery. A single band of the predicted size (477 bp) was consistently found from every renal structure (Fig. 3A). The Southern blots of the gels demonstrated specific binding of the

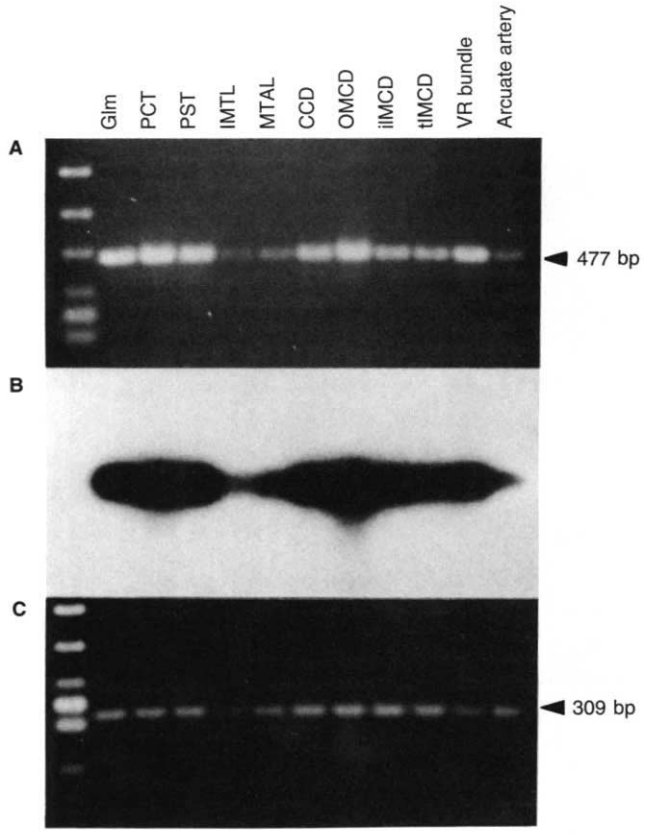

Fig. 3. *Detection of ET-3 mRNA in microdissected renal structures by RT-PCR.* (A) Ethidium bromide-stained agarose gel for ET-3. The arrow indicates expected PCR product size (477 bp). (B) Autoradiogram of corresponding Southern blot for ET-3. The blots were probed with a ^{32}P-labeled oligonucleotide that localized between the PCR primers. (C) Ethidium bromide stained agarose gel of GAPDH as a positive control. The arrow indicates expected PCR products size (309 bp). We obtained the same results from five experiments from five rats. Abbreviations are: Glm, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; IMTL, inner medullary thin limb; MTAL, medullary thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; iIMCD, initial inner medullary collecting duct; tIMCD, terminal inner medullary collecting duct; VR bundle, vasa recta bundle.

oligonucleotide probe to the 477 bp product (Fig. 3B). We confirmed that the PCR product was ET-3 cDNA by sequencing the subcloned PCR product.

Large signals for ET-3 were detected in OMCD, CCD, PCT, PST, Glm, and VR bundle. Small signals were found in iIMCD, tIMCD, MTAL, IMTL, and arcuate artery. Amplification product of GAPDH was detected from all renal structures at the predicted size (309 bp), and served as a positive control for the RT-PCR reaction (Fig. 3C).

Relative levels of the ET-3 amplification products among the nephron segments

The densitometry values are presented as percentages of the 2 mm OMCD values measured in the same experiment (Fig. 4). In PCT and PST, the signals were 61.9 ± 12.5% (vs. OMCD, N = 5) and 56.3 ± 6.3%, respectively. The values in IMTL and MTAL were 5.1 ± 2.5% and 9.6 ± 4.4%, respectively. Among

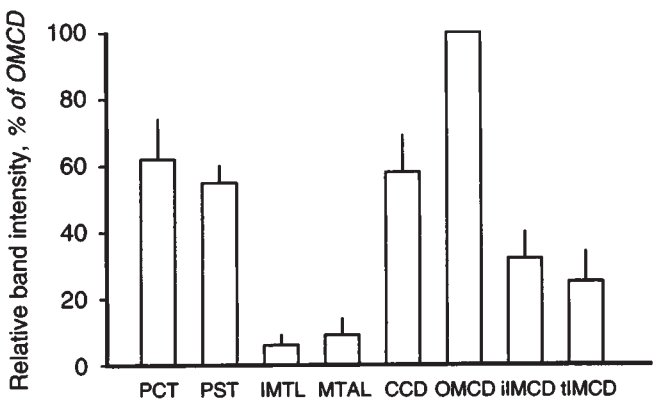

Fig. 4. *Relative quantitation of ET-3 mRNA in renal nephron segments.* The values for each segment are expressed as a percentage of OMCD values obtained in the same amplification run. The total number of each segment is five experiments from five rats.

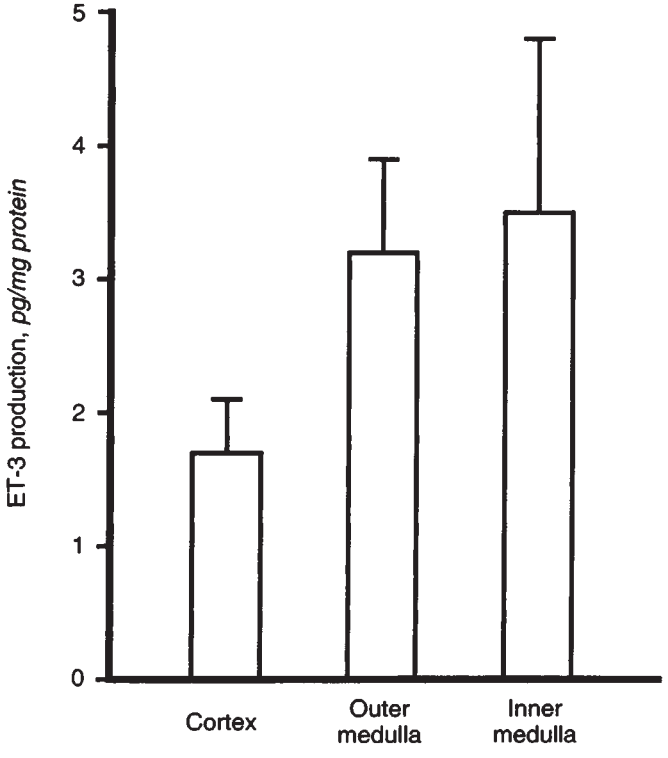

Fig. 5. *ET-3 production in tubule suspensions from cortex, outer medulla, and inner medulla of rats.* ET-3 in the supernatants of tubule suspensions from cortex, outer medulla, and inner medulla was measured using radioimmunoassay (N = 5, mean ± SEM).

collecting duct segments, the values were the following: CCD, 59. 7 ± 11.4%; iIMCD, 32.9 ± 7.0%; and tIMCD, 27.5 ± 9.1%.

ET-3 production by tubule suspensions of cortex, outer medulla, and inner medulla

ET-3 contents in tubule suspensions are shown in Figure 5. The amounts of ET-3 in tubule suspensions from inner medulla and outer medulla are 3.5 ± 1.3 and 3.2 ± 0.7 pg/mg protein (N = 5, mean ± SEM), respectively. Tubule suspension from cortex

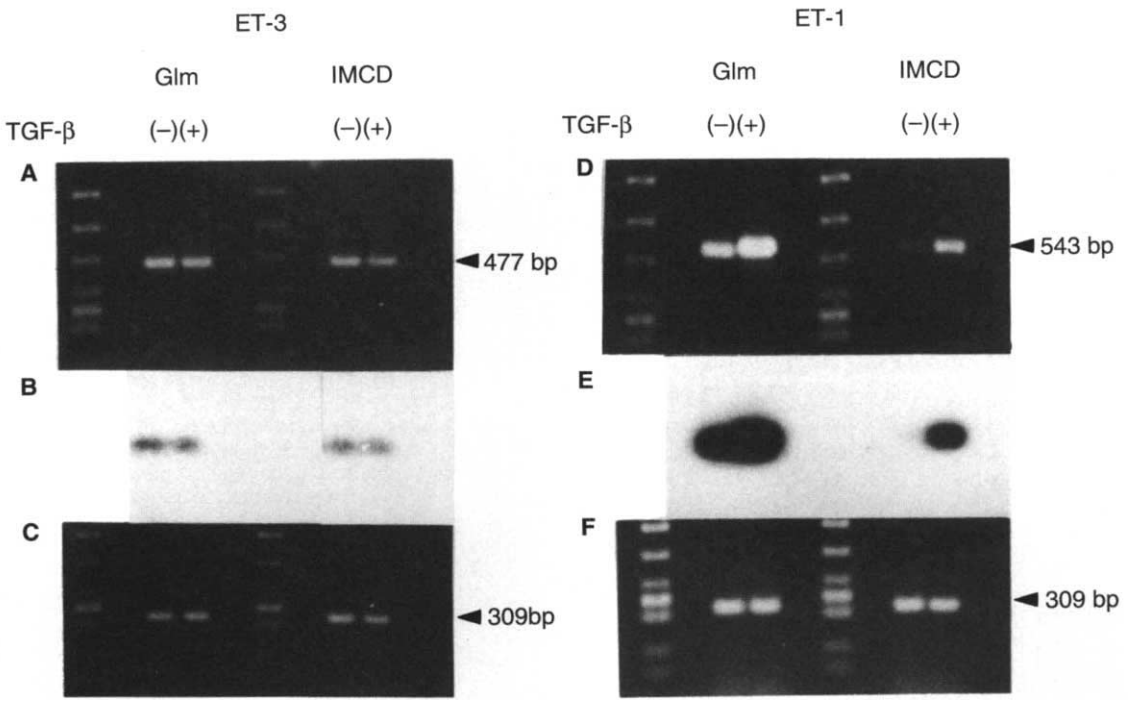

Fig. 6. *Effect of TGF-β on ET-3 and ET-1 mRNA expression in glomerulus and inner medullary collecting duct.* **A.** The result from agarose gel electrophoresis for ET-3 in Glm and IMCD. **B.** The corresponding Southern blot analysis. **D.** The result from agarose gel electrophoresis for ET-1 in Glm and IMCD. **E.** The corresponding Southern blot analysis. GAPDH is shown in **C** and **F** as a positive control. We obtained similar results from five experiments from five rats.

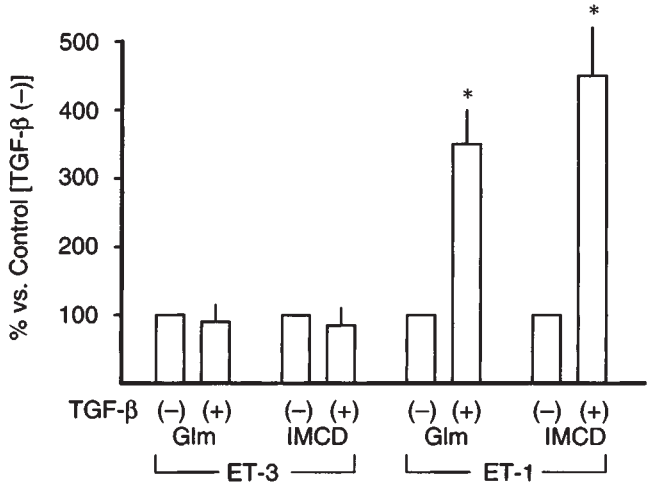

Fig. 7. *Densitometric analysis of the effect of TGF-β on ET-3 and ET-1 mRNA expression in glomerulus and inner medullary collecting duct.* This figure shows densitometry analysis of five experiments from five rats. Each bar represents mean ± SEM. We expressed the densitometric values of ET-1 or ET-3 incubated with TGF-β as a percentage the values of the sample incubated without TGF-β, and those values were also corrected by the densitometric value of GAPDH. ET-1 PCR products from Glm and IMCD incubated with TGF-β increased significantly from the samples incubated without TGF-β (* $P < 0.01$ vs. Control).

contains 1.7 ± 0.4 pg/mg protein. The levels of ET-3 from microdissected nephron segments (200 Glm or 100 mm of PCT, MTAL, OMCD, and IMCD) are below the detectable range.

ET-3 and ET-1 mRNA regulation by TGF-β in Glm and IMCD

Figure 6 A and B show a typical agarose gel and corresponding Southern blot of ET-3. Incubation with or without TGF-β in the presence of 0.5% FCS caused no significant change of ET-3 PCR product, both in Glm and IMCD. On the other hand, ET-1 PCR product showed a significant increase in the presence of TGF-β in both Glm and IMCD (Fig. 6 D and E), as we reported previously [5]. The internal standard, GAPDH, showed almost the same intensity with and without TGF-β (Fig. 6 C and F). We obtained similar results from five experiments from five rats. Figure 7 shows densitometry analysis of each experiment. We expressed the densitometric values of ET-1 or ET-3 incubated with TGF-β as a percentage of the values of the sample incubated without TGF-β, and these values were corrected by the densitometric value of GAPDH. The percentages of ET-3 PCR products from Glm and IMCD incubated with TGF-β are 92.3 ± 20.1% ($N = 5$, mean ± SEM), and 88.6 ± 32.3%, respectively (Fig. 7). The percentages of ET-1 PCR products from Glm and IMCD incubated with TGF-β increased significantly, to 351.7 ± 52.0% ($N = 5$, mean ± SEM) and 446.1 ± 70.3%, respectively (Fig. 7). Thus TGF-β regulated the expression of ET-1 mRNA, but not ET-3 mRNA, in our experimental condition.

Discussion

The main result of our study is that almost every nephron segment and the renal vascular system express ET-3 mRNA.

Incubation with TGF-β did not change the ET-3 PCR signal, whereas the ET-1 PCR signal was increased significantly by exposure to TGF-β in Glm and IMCD. We found especially high mRNA levels in OMCD, PCT, CCD, PST, and Glm. Thus, ET-3 and ET-1 are distributed differently along the nephron and are regulated in different manners. We detected ET-3 protein from tubule suspensions from cortex, outer medulla, and inner medulla. Our results show that ET-1 and ET-3 mRNA are regulated differently in Glm and IMCD. ET-1 and ET-3 may play different roles in modulating renal function, because their localization and regulation are different. In the following sections, we will discuss the potential role of ET-3 in the kidney.

To our knowledge, this is the first report which clearly demonstrates that ET-3 mRNA is expressed in intact Glm. Although Kasinath et al, using immunocytochemistry, reported that cultured glomerular epithelial cells synthesized ET-3 [19], it had not been shown whether intact Glm expressed ET-3 mRNA. In isolated perfused rat kidney, ET-3 markedly increased GFR, fractional excretion of water and electrolytes, and urinary excretion of PGE_2 and PGF_2 [6]. Yamashita et al also reported that continuous infusion of ET-3 increased RBF and urine flow rate, presumably via enhanced renal production of NO induced by ET-3 [7]. We previously reported that ET-BR, constitutive NO synthase, and soluble guanylate cyclase mRNAs were expressed in Glm, arcuate artery, and VR bundle [8, 20]. In addition, ET-3 stimulates cGMP production in isolated rat glomerulus (21). Therefore, ET-3 that is synthesized in Glm, arcuate artery, and VR bundle may act in an autocrine or paracrine fashion and stimulate ET-BR, and may regulate RBF and medullary blood flow via NO, PGs, and cGMP synthesis.

Firth and Ratcliffe reported that ET-3 mRNA expression was at relatively high levels in the kidney [9]. Our data demonstrate that in collecting ducts, especially in OMCD, a relatively high amount of ET-3 mRNA is expressed. Kohan had demonstrated that the largest ET-3 production was found in IMCD cells, but he did not examine OMCD cells [11]. We also detected ET-3 protein in tubule suspensions from outer medulla and inner medulla. The reason we could not detect ET-3 protein from microdissected OMCD or IMCD is because the lower limit of sensitivity for ET-3 measurement is around 1 pg. To collect several mg protein from microdissected tubule is not practical, because it requires more than 10,000 mm lengths of tubules.

Urine flow increases approximately twofold during ET-3 infusion, and urinary osmolality decreases markedly [7]. This suggests that ET-3 modulates the function of the distal nephron. Our previous report showed that ET-BR mRNA was expressed in collecting ducts [8]. Thus ET-3 synthesized locally may bind to ET-BR in the collecting ducts in an autocrine or paracrine fashion and influence water and electrolyte transport. In these segments, ET-1 is already known to influence vasopressin-induced cAMP levels and suppress water permeability [22]. ET-3, like ET-1, could influence the function of collecting ducts via ET-BR.

Our data show that PCT and PST express large amounts of ET-3 mRNA. Kohan showed that the ratio of ET-3 to ET-1 accumulation was greatest in proximal tubule cells [11]. Our previous report demonstrated that ET-1 mRNA could not be detected in PCT [5]. These results suggested that proximal tubules synthesize mainly ET-3 rather than ET-1.

Our results demonstrated that ET-1 mRNA increased in the presence of TGF-β in Glm and IMCD, whereas we could not detect an ET-3 mRNA change by incubation with TGF-β. Our experimental condition is relatively specific for seeing the gene regulation, because we used intact Glm or IMCD in a six hour incubation. We have not done other experiments using a cultured cell system or different incubation conditions. Thus, it seems difficult to conclude whether or not ET-3 gene transcription is regulated by TGF-β only from our observations. The 5′ flanking region of the ET-1 gene contains the consensus sequence of the binding site of nuclear factor-1, which may mediate induction by TGF-β [23]. On the other hand, information on the 5′ flanking region of ET-3 gene is not yet available.

Our approach to the relative quantitation of the ET-3 PCR amplification product was to run Southern blots of the amplified cDNA using a ^{32}P-labeled hybridization probe, and then to assess the amount of bound radioactivity by densitometry of the resulting autoradiograms. OMCD consistently gave a large signal that was relatively invariant between replicates from experiment to experiment. Therefore we used the signal of OMCD as a control and expressed the densitometry values of other segments as percentages of OMCD. In preliminary experiments, we compared the methods of mRNA extraction. In our case, when we extracted RNA using the acid guanidinium thiocyanate-PhOH-chloroform method [24] or mRNA purification kits (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA), the efficiency of RNA extraction was not stable between samples, thus the amount of PCR product was not consistent. We believe that our method using Triton X-100 is the best way to avoid instability of this step. We also compared the quantitative quality of the PCR method in several PCR cycles in a preliminary experiment. Although we observed log-linear phase till 40 cycles, we selected only 30 cycles for ET-3 amplification. If we amplified 35 cycles, the amount of PCR products became large, but the accuracy of quantitative analysis became lower, even though the PCR is log-linear phase.

We chose a simple approach to relative quantitation rather than any of the complicated alternatives, such as competitive PCR [25] and PATTY (PCR aided transcript titration assay) [26], for several reasons. The ability of any method to yield absolute quantitation remains in question and would have to be carefully documented. The value of knowing the absolute number of mRNA molecules present in a given cell may be extremely limited for most physiological questions, because there is presently no way to relate this number to the ultimate functional expression of the gene product.

We believe that our method gives a relative measure of the amount of ET-3 mRNA initially present in the cells. This conclusion is based on the observation that there is an approximately linear relationship between the lengths of tubules used for the assay (that is, the amount of starting material) and the value obtained from densitometry of the amplification product band.

In summary, our data show that ET-3 mRNA distributes widely along the nephron and vascular system, and large signals were detected in PCT, PST, CCD, OMCD, and Glm. ET-3 mRNA does not change significantly in the presence or absence of TGF-β. ET-3 mRNA distributes and may be regulated differently from ET-1 in the rat kidney. ET-1 and ET-3 may play different roles in modulating renal functions.

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